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STUDIES ON THE DIFFERING EFFECTS OF TUMOR NECROSIS FACTOR AND LYMPHOTOXIN ON THE GROWTH OF SEVERAL HUMAN TUMOR LINES

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The relative ability of TNF and lymphotoxin (LT) t inhibit the growth of five human tumor cell lines was examined both in the presence and absence of IFN-γ. Two adenocarcinoma lines, HT-29 and SK-CO-1, were 20- and 320-fold more sensitive to the inhibitory effects of TNF than LT in 3- to 4-day proliferation assays. In contrast, the breast carcinoma line BT-20 showed only a one- to twofold difference. The MCF-7 and ME-180 cell lines exhibited intermediate behavior. These results parallel the reported disparate potencies of TNF and LT in their effects on endothelial cells, hematopoietic development and their abilities to sustain a mixed lymphocyte response. Radiolabeled TNF binding studies showed two classes of receptors (K_d 0.04 to 0.15 nM and 0.2 to 1.0 nM) on the highly sensitive SK-CO-1 line. HT-29 cells also appeared to possess some high affinity-binding sites, whereas the BT-20 line completely lacked the high affinity form. Thus the presence of high affinity-binding sites correlated with increased sensitivity to the antiproliferative effects of TNF. Cold TNF competed with the binding of radiolabeled human TNF three- to fivefold better than LT for binding to all three lines. These relatively small differences between the TNF and LT receptor-binding characteristics are insufficient to explain the dramatic disparity in their antiproliferative properties. Likewise, the absolute concentrations of the unlabeled cytokines necessary to block the binding of ^{125}I-TNF were 10- to 150-fold higher than the levels necessary to elicit the biologic response. Thus, the receptor binding data conflict with the growth inhibitory effects. This discrepancy is discussed in terms of either separate receptors for TNF and LT or more complex phenomena such as receptor cooperativity possibly resulting from multivalent interactions with the trimeric form of TNF.

TNF and LT[2] also called TNF-α and TNF-β, respectively, are two proteins characterized originally for their cytolytic properties and, in the case of TNF, its in vivo tumor necrotic activity (for reviews see References 1 to 5). These proteins can be directly cytolytic to certain tumor lines, yet in other cases they can actually promote growth (6, 7). TNF is produced by many cell types including monocytes, T cells, NK cells (1, 9), and even cells of nonhematopoietic origin (8, 10), whereas LT appears to be released primarily by lymphocytes (2, 11–13). More recently, the cloned gene products have been demonstrated to display a wide range of activities. TNF appears to play major roles in specific aspects of metabolic control, the response to endotoxin shock (14), the control of hematopoietic cell development (15–18), and it shares with IL-1 many proinflammatory actions (e.g., Reference 19). Although many of these activities have also been demonstrated for LT, a comprehensive picture of their relative roles remains unclear.

Initially TNF and LT were described as having similar cytotoxic activities (20), however, more recent work has indicated that LT is less active on certain tumor lines (21, 22). A comparison of LT and TNF activities reveals disparate potencies in several systems. The human endothelial cell responds to TNF stimulation by secreting IL-1 and CSF, by expressing surface adhesion proteins, and by exhibiting increased adherence for neutr phils (23, 24). Similarly, granulocyte CSF release from fibroblasts was induced differently by LT and TNF (25). In each of these cases, LT was at least 100-fold less active than TNF. In our experiments, TNF was 20- to 50-fold more effective than LT in its ability to induce primary human umbilical vein endothelial cells to bind retinoic acid-induced HL-60 cells (J. Browning and P. Lawt n, unpublished results). Likewise, LT is less effective relative to TNF in inducing human peripheral monocytes to release CSF-1 (18) and in its ability to promote the mixed lymphocyte response (26). In contrast to this pattern, LT has been reported to cause an inflammatory reaction in normal skin whereas TNF was inactive (27). In receptor-binding experiments with ME-180 (28), L929 (29, 30), HL-60 (31), and U937 (32, 33) cells, LT competed with labeled TNF almost equally well for binding to its rec ptor and vice versa, suggesting that both molecules interact with the same or similar receptors. The conflicting nature of the observations that these proteins share common receptors yet display widely differing activity profiles poses an interesting problem.

In this paper, we examined the anti-proliferative effects of TNF and LT on several human tumor lines both in the presence and absence of the synergistic factor, IFN-γ and have attempted to correlate differing biologic activities with receptor-binding properties. The apparent discrepancy between the receptor-binding studies and the biologic effects is discussed.

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[2] Abbreviations used in this paper: LT, lymphotoxin; MTT, 3(4,5-dimethylthiazol-2-yl)2,5 diphenyl tetrazolium bromide; EGS, ethylene glycol bis(succinimidylsuccinate); CHO, Chinese hamster ovary.

MATERIALS AND METHODS

Cytotoxins. Human rTNF was expressed in *Escherichia coli* and purified to homogeneity as previously described (34). This preparation had a specific activity of 5.3×10^7 U/mg as defined by reference to the National Biological Standards Board TNF (Hertfordshire, England) in the 1-day L929 cell cytolytic assay. Early experiments with murine rTNF utilized protein produced at Biogen, whereas later experiments employed material that was a gift of Suntory Pharmaceutical Co. (Osaka, Japan). Both preparations had a specific activity of 3×10^7 U/mg in the L929 assay system. A cDNA clone encoding human LT was isolated from a RPMI 1788 cDNA library by using an exact oligonucleotide probe to the sequence previously described (20). The gene was stably transfected into CHO cells and amplified by using a dihydrofolate reductase/methotrexate selection system. rLT was purified from serum-free, LT-transfected CHO cell-conditioned media with a series of Sepharose S, lentil lectin, and FPLC Mono Q column chromatography steps. rLT purified by this route was 90 to 95% pure, was glycosylated, and had a specific activity of 3.1×10^7 U/mg by reference to the standard TNF sample. The protein content of the TNF and LT samples was determined by parallel amino acid composition analysis. Aliquots of cytotoxins at 1 (LT) and 10 (TNF) μg/ml were frozen and fresh aliquots thawed for each assay. Human rIFN-γ was produced from *E. coli* with a specific activity of 1.5×10^7 U/mg (encephalomyocarditis virus/WISH cell system). Murine rIFN-γ was produced in *E. coli* yielding a preparation with a specific activity of 1×10^7 U/mg by using a vesicular stomatitis virus/L929 assay system.

Cytolytic and cytostatic assays. The murine tumor line L929 and the human lines HT-29, SK-CO-1, BT-20, ME-180 and MCF-7 were obtained from the American Type Culture Collection (Rockville, MD) and grown in RPMI 1640 supplemented with glutamine, 50 μM 2-ME, penicillin/streptomycin, pyruvate, 10 mM HEPES buffer, and 10% Hyclone FCS (L929, HT-29, and ME-180 lines) or MEM (Eagle's) supplemented as above only without HEPES and with nonessential amino acids (SK-CO-1, BT-20, and MCF-7 lines). The cells were free of mycoplasma contamination (Gen-Probe test) and periodically monitored. One-day cytolytic assays using murine L929 cells were carried out by preplating L929 cells in 96-well plates (0.05 ml/well) before use. When the cells were about 50% confluent (1 to 2 days), serial dilutions of TNF or LT in 0.05 ml with 2 μg/ml mitomycin C were added. Growth was quantitated by using mitochondrial reduction of the dye MTT as described (35). After 20 to 30 h, 0.01 ml of 5 mg/ml MTT dye was added, and after an additional 3 to 4 h, 0.1 ml of isopropanol with 10 mM HCl was added to dissolve the reduced MTT dye. The OD at 570 nm was determined with an ELISA plate reader. Cytostatic assays of 3- to 4-day duration were performed as described for the cytolytic assay except that mitomycin C was omitted. Typically, wells were seeded with 3000 to 8000 cells/well at day 0, which resulted in a 0.4 to 0.8 OD signal after 3 to 4 days of growth. The initial cell concentration was adjusted to compensate for the slow growth of lines such as SK-CO-1 and BT-20. After 3 to 4 days, growth was assessed by using the MTT dye reduction method as described above. Although the absolute concentration of cytotoxin required to give 50% inhibition varied slightly from experiment to experiment, the potency of LT relative to TNF was very reproducible. In both the growth-inhibition assays and the receptor-binding studies, a monomeric m.w. was assumed for both TNF and LT.

TNF-binding assays. rTNF was iodinated by the Iodogen method as described (28) except that the following ratios of reactants were used: 2 μg Iodogen, 10 μg TNF, and 1 mCi of ^{125}I in a volume of 0.05 ml. ^{125}I was reacted with Iodogen alone for 2.5 min at 0°C followed by the addition of TNF and the reaction continued for 10 min. The free iodine was separated on a BioRad P-6DG column. Bioassay using L929 cytotoxicity showed typically retention of at least 50% of the starting activity yielding a specific activity of 12 to 20 μCi/μg active TNF. SDS-PAGE and autoradiography showed that only TNF was iodinated. For direct binding experiments, cells from nonconfluent plates were removed with 5 mM EDTA in calcium/magnesium-free PBS, collected and resuspended at a concentration of 0.5 to 2×10^7 cells/ml by repeated passage through a 5-ml pipette into RPMI 1640 with 10% FCS and 0.1% sodium azide. Aliquots of 0.5 ml were rocked in a 3-ml conical polypropylene tube for 4 h at 4°C in the presence of ^{125}I-TNF. Nonspecific binding was assessed by including 4 μg/ml cold TNF in control samples and it constituted generally 20% or less of the total binding. The cells were washed twice with 3 ml of ice-cold Dulbecco's PBS with 5% FCS and 0.1% sodium azide and counted. Direct binding was analyzed with a standard Scatchard plot. Competitive binding was analyzed in a similar fashion by using 50 pM ^{125}I-TNF in the case of HT-29 and SK-CO-1 cells and 100 pM with the L929 cells. The same unlabeled TNF and LT solutions used in the growth inhibition assays were employed in the competition assays. Competitive binding was analysed by using logit analysis whereby logit = ln $(Y/1-Y)$ and Y is equal to percent bound (36). In all of the receptor-binding analyses, curves were fitted by eye and no attempt was made to deconvolute curvilinear Scatchard plots by more rigorous methodologies.

TNF/LT cross-linking. ^{125}I-TNF or ^{125}I-LT (prepared as described above for TNF) was diluted into 50 mM potassium phosphate buffer, pH 7.4, with 100 μg/ml BSA and with or without various detergents. Samples at final concentrations of 100 (TNF) and 50 (LT) ng/ml were treated at 0°C with 1 mM EGS (Pierce Chemical Co., Rockford, IL; a concentrated stock solution was prepared in dry dimethylformamide) or at room temperature with glutaraldehyde (Electron Microscopy Sciences, Ft. Washington, PA) at a final concentration of 0.1% v/v. After 30 min, SDS-gel sample buffer was added and the samples were boiled and analysed by SDS-PAGE and autoradiography.

RESULTS

Properties of CHO-derived human lymphotoxin. The amino acid sequence of human LT as determined from the cDNA nucleotide sequence agreed completely with the previously published sequence (20). Because the properties of rLT secreted from CHO cells have not been well described, we have partially characterized this protein. On the basis of lentil lectin binding, the bulk of the secreted LT was glycosylated. The major rLT species migrated in SDS PAGE at an apparent M_r of 20 kDa (see Fig. 1), which is greater than the 18-kDa nonglycosylated form produced in *E. coli* (20), yet smaller than the 25-kDa species originally purified from the human B-lymphoblastoid line, RPMI 1788 (37). The amino acid composition of the purified protein agreed with the expected composition. Initial batches of rLT contained two minor species of slightly higher of M_r. *N*-terminal amino acid sequencing from polyvinylidene difluoride blotted (38) samples of all three forms revealed identical *N* termini corresponding to the *N* terminus of the 25-kDa form described by Aggarwal et al. (37). The different forms probably result from heterogeneous glycosylation. A set of LT-like proteins can be immunoprecipitated from ^{35}S-methionine-labeled, phorbol ester/PHA-stimulated PBL with a rabbit antiserum raised against the purified CHO cell-derived LT (unpublished observations). In this case a ladder of presumably different glycosylated forms was observed and the predominant form secreted from CHO cells corresponded to the smallest form produced by peripheral lymphocytes. The major species released by lymphocytes migrated at 25 kDa. In an examination of three HIV-infected human T cell lines, three different LT sizes were observed (39). Apparently, most of the natural lymphocyte-derived LT is more heavily glycosylated than CHO-derived rLT. A SDS-PAGE gel comparing recombinant *E. coli*-derived TNF with rCHO cell-derived LT is shown in Figure 1. The LT preparation was 90 to 95% pure with the major impurity being a 17-kDa protein corresponding probably to the 20-kDa form described by Aggarwal et al. (37).

TNF exists as a compact trimer in solution (40, 41). Superose S6 gel exclusion FPLC chromatography in 0.1 mg/ml BSA, 0.2 mM EDTA and PBS showed that both human and murine rTNF migrated with an apparent M_r of about 45 kDa. CHO cell-derived human rLT also migrated with a M_r of 45 kDa in this system. The iodinated preparations of TNF used in the binding studies co-migrated with unlabeled TNF. Cross-linking studies were carried out to establish more rigorously whether LT is a trimer. Figure 2 shows the results of EGS and glutaraldehyde cross-linking of iodinated TNF and LT. EGS was capable of almost completely cross-linking TNF into a

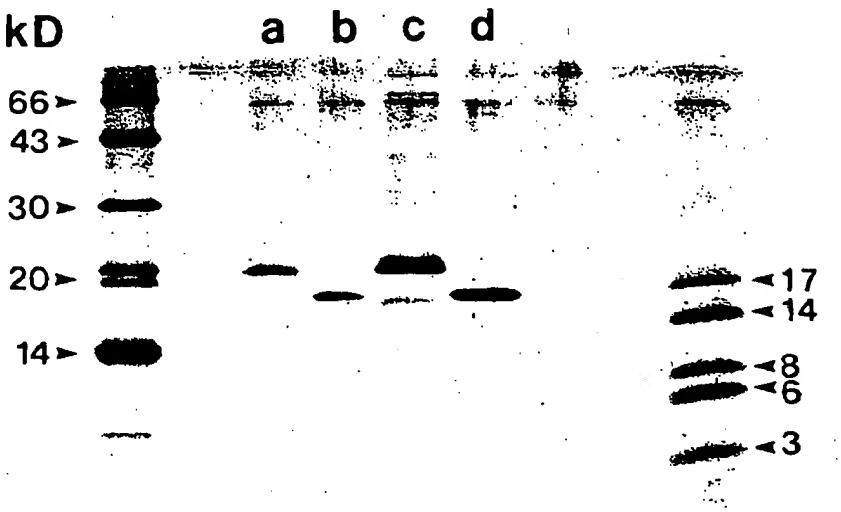

Figure 1. SDS polyacramide gel comparison of human rLT and human rTNF. Lanes were loaded with 0.2 μg (*a, b*) or 1.0 μg (*c, d*) of LT (*a, c*) or TNF (*b, d*). Gel was composed of 16% acrylamide/0.49% bis acrylamide and run according to the system described by Schaegger and Jagow (63) and stained with Coomassie blue. Bands at 68 kDa represent fingerprint artifacts.

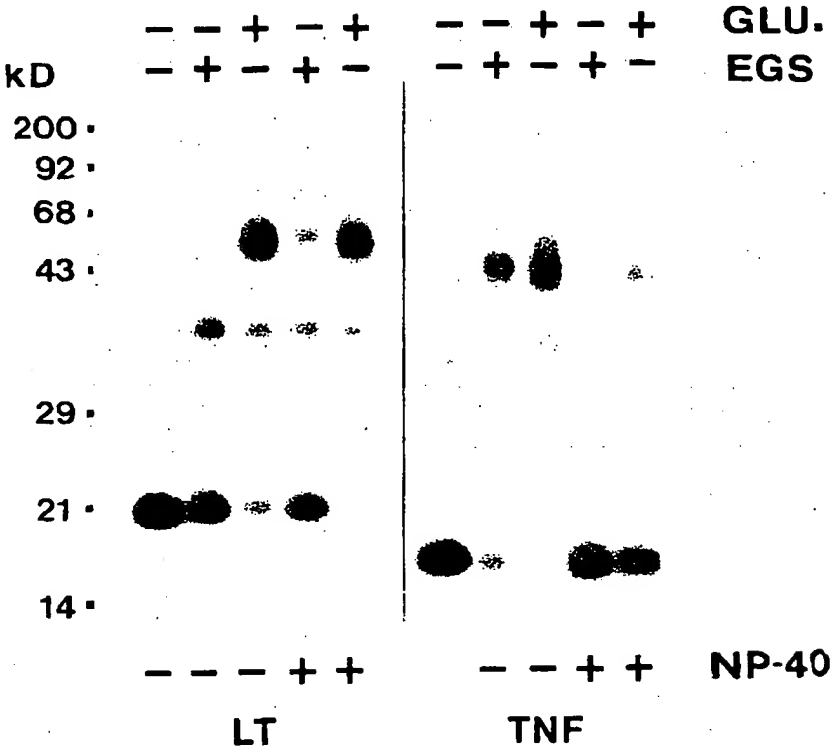

Figure 2. Reduced SDS polyacrylamide gel analysis of either EGS or glutaraldehyde (GLU.) cross-linked preparations of iodinated LT and TNF. Cross-linking was performed in the presence or absence of 1% NP-40. Shown is an autoradiograph of the gel. The m.w. standards were Bethesda Research Laboratory-prestained standards and the indicated sizes are the values as assessed by running these markers alongside the markers used in Figure 1.

trimeric structure and with LT both dimeric and trimeric forms were observed. LT is cross-linked less efficiently by EGS. Glutaraldehyde was more effective than EGS in cross-linking both TNF and LT and both glutaraldehyde-treated cytokines were found as a trimers with no indications of larger aggregates. TNF cross-linking was completely blocked by inclusion of 1% NP-40 in agreement with the reported disruption of the TNF trimeric structure by detergents (41). In contrast, the trimeric structure of LT is stable to detergent treatment. The cross-linking of LT by both EGS and glutaraldehyde was actually enhanced by addition of either 1% NP-40 or 0.1% SDS (never warmed beyond room temperature). Thus LT is a very stable trimer, even at concentrations approaching physiologic levels, and this result is in agreement with the trimeric m.w. reported for natural RPMI 1788-derived LT (42).

Specific activities of LT and TNF for cytolysis and cytostasis of L929 cells. A comparison of TNF and LT in both 1- and 3-day murine L929 proliferation assays is shown in Figure 3. LT was slightly less potent than TNF on a molar basis in the 1-day assay with 50% lysis observed at 28 to 33 pM for LT and 13 to 15 pM for TNF. On the other hand, LT was more effective in the 3-day assay (Fig. 1 and Table I) similar to results previously reported (43). The reasons for the increased potency in the 3-day assay are not clear. In the 3-day L929 antiproliferative assay, the potency of TNF also increased about twofold. The specific activity for this preparation of LT is similar to the values determined for both natural and *E. coli*-derived recombinant material as assessed by using the 1-day assay (20), thus glycosylation of the protein by the CHO cell does not affect the activity as was found previously for natural and *E. coli*-derived recombinant

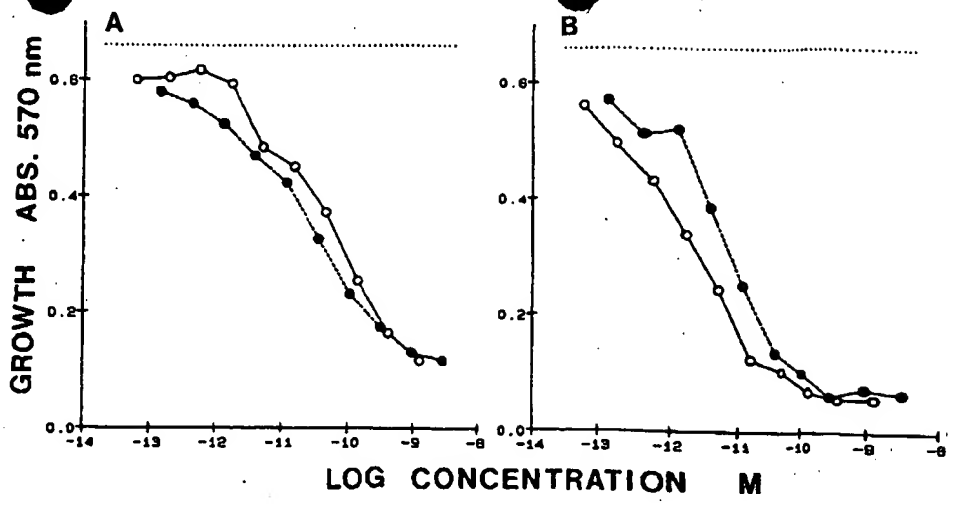

Figure 3. Comparison of recombinant human LT (O——O) and recombinant human TNF (●----●) in 1-day cytolytic (*A*) and 3-day anti-proliferative (*B*) assays using the murine fibrosarcoma L929. In the cytolytic assay about 20,000 cells/well were present, whereas in the 3-day assay, wells were seeded with 3,000 cells/well. Growth was quantitated with the MTT reduction system and relative growth is presented as OD_{570} nm. Dotted lines at the top of the panels represent the levels of growth observed in the absence of added rTNF or rLT.

TABLE I

Effects of LT and TNF on the growth of several tumor lines

Cell Line	Expt. No.	Concentration (pM) Giving 50% Growth Inhibition			
		−IFN-γ		+IFN-γ[a]	
		LT	TNF	LT	TNF
L929 (1-day)[b]	1	33	13	35	12
	2	26	15		
	3	28	14		
L929 (3-day)	1	3	12		
	2	1	4		
	3	3	8		
HT-29	1	>2500[c]	>5000	610	31
	2	>2500	>5000	372	16
SK-CO-1	1	>2500	>5000[d]	299	1
	2	>2500	>5000	300	2
BT-20	1	667	368	179	137
	2	415	285	139	106
ME-180	1	1250	184	215	13
	2	2500	552	240	33
MCF-7	1	>2500	2000	>2500	2000
	2				

[a] Murine IFN-γ was used in the case of L929 cell assays, human IFN-γ was used in all other cases. Both IFN were added at a concentration of 500 antiviral U/ml at the beginning of the assay.

[b] One-day cytolytic assay as opposed to 3-day growth assays in all other cases.

[c] The use of the greater than symbol indicates that the 50% point was not reached at this concentration.

[d] TNF stimulated growth in the absence of IFN-γ.

forms (20).

Specific activity of LT and TNF for cytostasis of several human tumor lines. IFN-γ synergizes with TNF and LT in antiproliferative assays (44–46). The effects of TNF and LT on the growth of five TNF-sensitive human tumors was examined both in the presence and absence of IFN-γ. The results of three such comparisons are shown in Figure 4 and summarized in Table I. The tumors differed dramatically both in the absolute sensitivity to TNF and LT as well as in the relative potency of these tw proteins. For example, HT-29 cells have been described as being very sensitive to TNF in the presence of IFN-γ (46, 47) as was observed in these studies. In the absence of IFN-γ, HT-29 cells are unaffected by ven very high levels of TNF. We had noticed during an earlier investigation of the lymphotoxin-like cytotoxins produced by the RPMI 1788 and the GM 3104 cell lines that what appeared to be natural lymphotoxin had a low specific activity on IFN-γ-treated HT-29 cells. This observation was confirmed with CHO-derived rLT in that rLT was about 20 to 25 fold less potent than TNF both on a molar basis and in terms of L929 LU. The SK-CO-1 line behaved in a fashion similar to the HT-29 adenocarcinoma in that IFN-γ was absolutely required for antipr - liferative activity of LT or TNF. IFN-γ alone exhibited slight antiproliferative effects on the HT-29, SK-CO-1, and BT-20 lines at the 500 U/ml level. In the absence of IFN-γ, TNF acted as a growth factor on SK-CO-1 cells, an observation typically made in fibroblastoid systems (6, 7). Thus IFN-γ changes the way this cell interprets the signals generated by TNF, i.e., from enhanced growth t growth arrest responses. One could speculate that the antiproliferative action of IFN-γ is a result of an altered response to growth factors provided by the serum in a manner analogous to the TNF effects on the SK-CO-1 line. The antiproliferative activity of TNF was 200 to 320 times more potent than that of LT with this cell line. Although the degree of growth stimulation by TNF in the absence of IFN-γ was small at least as quantitated with the MTT assay, the concentration dependence paralleled closely that of the cytostatic activity observed in presence of IFN suggesting that IFN-γ does not modulate the affinity of the TNF receptor. A lack of receptor-affinity modulation by IFN-γ has been reported in other systems (46, 47). We attempted to block the growth inhibition of SK-CO-1 by 1 pM TNF with 50 to 100 nM LT. LT alone at these concentrations did not affect growth and its inclusion with TNF did not block the TNF activity. Hence, LT did not act to antagonize the low level TNF effect.

The breast carcinoma line, BT-20, was responsive to both LT and TNF in the absence of IFN-γ (IFN-γ being only slightly synergistic) and TNF was only one- to two-fold more potent than LT. The cell line ME-180, a cervical carcinoma, exhibited intermediate behavior with some synergistic interaction with IFN-γ and a 20-fold higher potency of TNF over LT. The breast carcinoma line, MCF-7, in our hands was relatively unresponsive to TNF with no synergy with IFN-γ. The 50% point for growth inhibition by LT f r f ur of these lines remained in the 200- to 400-pM range. In contrast, the 50% point for TNF effects varied between 1 and 150 pM.

Receptor-binding studies. Scatchard analyses of di-

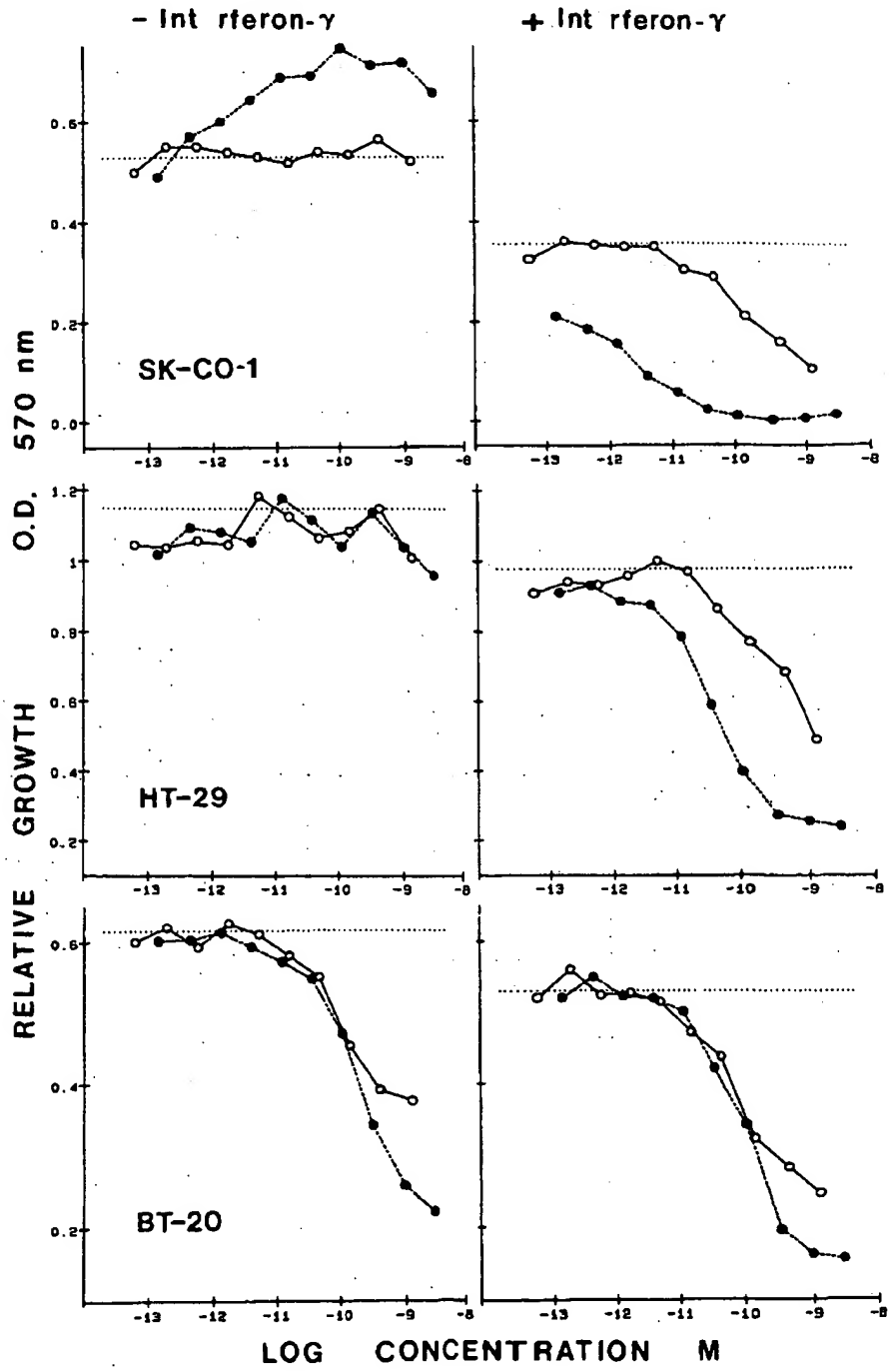

Figure 4. Comparison of the antiproliferative activity of rLT (O——O) and rTNF (●-----●) on the growth of three human tumor lines: SK-CO-1, HT-29, and BT-20. Wells were seeded with 8000 (SK-CO-1 and BT-20) and 4000 (HT-29) cells/well and growth was assessed after 3 days (HT-29 and SK-CO-1) or 4 days (BT-20). Cultures were set up either with or without 500 U/ml of IFN-γ. Dotted line signifies the level of growth observed in the absence of rTNF or rLT, either with or without IFN-γ.

rect ^{125}I-TNF-binding experiments are shown in Figure 5 and summarized in Table II. In these studies, the concentration of active TNF was quantitated by using the L929 bioassay and specific activities were calculated, assuming that both inactive and active molecules were labeled uniformly. In one case the self displacement method was used to assess the sp. act. and a roughly similar value was obtained (48). The L929 cell line bound 2500 to 5500 m lecules f human TNF per cell with a dissociati n constant of 0.5 to 1.0 nM, a value that agrees well with previously published results (30, 49). In one set of exp r-iments, the Scatchard plots had clearly defined concave downward appearances when data at low TNF concentrations were included (data not shown). Such plots can be indicative of positive cooperativity (50). Scatchard analysis of TNF binding to SK-CO-1 cells revealed two types of binding sites, a low affinity form with 3000 to 6000 sites per cell and a high affinity form with 1000 to 2000 sites/cell. The dissociation constant of the high affinity form, K_d 0.07 to 0.15 nM, was 5- to 10-fold higher than that of the low affinity form, K_d 0.2 to 1.0 nM. The HT-29 Scatchard plot was curvilinear, probably a result of contributi ns by both a low affinity site similar to that observed on the SK-CO-1 line and some higher affinity components. The number of receptor sites increased slightly with a 6-h pretreatment with IFN-γ, however, these increases were not observed with 24- to 48-h exposure parall ling previous reports (46, 47). The BT-20

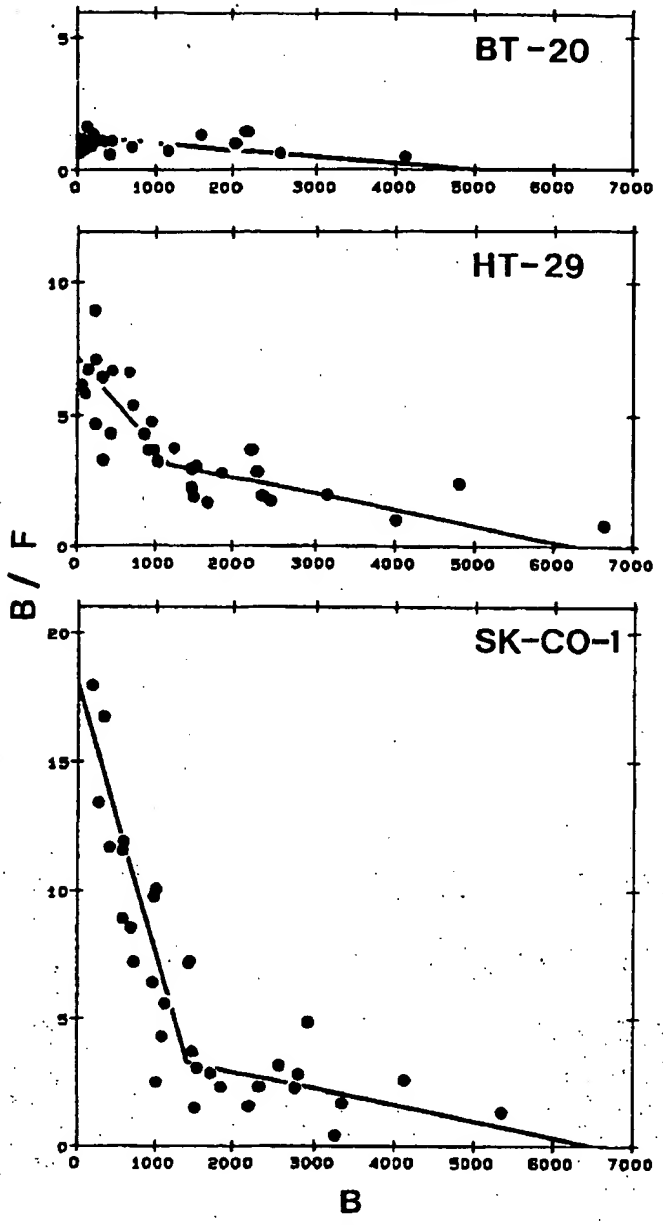

Figure 5. Scatchard analyses of the direct binding of ^{125}I-TNF to three human tumor lines. *B* represents number of TNF molecules per cell. Shown are data pooled from several experiments, and in each case the cells had been treated with IFN-γ for 6 to 48 h.

TABLE II

Summary of Scatchard analyses of direct ^{125}I-TNF binding to various tumor lines

Cell	−IFN-γ		+IFN-γ[a]	
	Sites/cell	K_d (nM)[b]	Sites/cell	K_d (nM)
L929	2500–5500	0.61–0.80		
BT-20			3000–5000	3.30–6.50
HT-29	2000–3300	0.25–1.00	1100–6000	0.40–1.10
SK-CO-1				
Low[c]	3000	0.46	3000–6500	0.20–1.00
High	1500	0.07	600–2000	0.04–0.15

[a] Pretreatment with 1000 U/ml IFN-γ for 6 or 48 h.
[b] Data represent the range of all experiments.
[c] Indicative of the high and low affinity sites found with this cell line.

line possessed the lowest affinity receptors and clearly lacked any high affinity receptor forms. Thus increased sensitivity to the biologic effects of TNF correlated with the presence of apparently higher affinity receptors.

Logit plots of pooled data from competitive binding experiments are sh wn in Figure 6 and the results of individual experiments summarized in Table III. Figure 6 represents data from both multiple cell preparations and different iodinated TNF lots. Both LT and TNF competed with labeled TNF binding to all the cell types examined. In the case of the murine L929 cell, human LT was similar to murine TNF and both were more effective than human TNF. Whether this increased binding of LT over TNF simply reflects aspects of the species barrier or implies that the L929 receptor is actually a LT receptor is not clear. In contrast to the murine cell line, LT was three- to fivefold less effective than TNF in competing with labeled TNF binding to the human HT-29 and SK-CO-1 lines. The BT-20 line possesses relatively low affin-

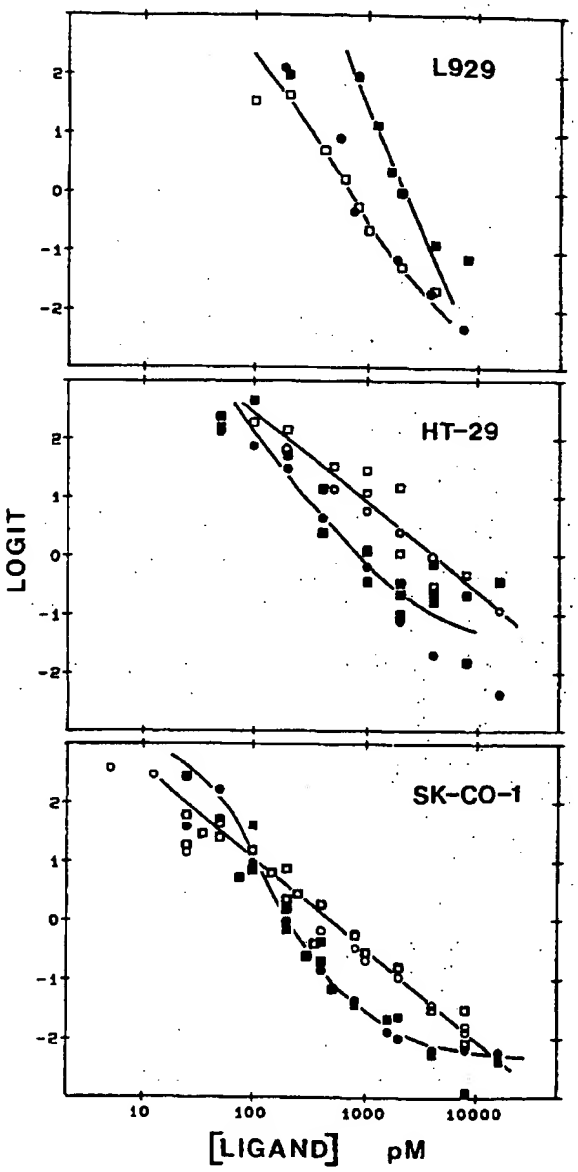

Figure 6. Logit analysis of the competition of unlabeled human rLT (*open symbols*) or unlabeled human rTNF (*closed symbols*) with ^{125}I-human TNF for binding to murine L929, human HT-29, and human SK-CO-1 cells. In the experiments with HT-29 and SK-CO-1 cells, data from several experiments have been pooled and different symbols were used to denote whether the cells were pretreated with IFN-γ (*squares*) or were untreated (*circles*). In the L929 cell panel, the *closed circles* represent competition by unlabeled murine rTNF and the cells were not exposed to IFN-γ.

TABLE III
Summary of competitive binding experiments

Cell Line	Concentration of Unlabeled Ligand (pM) at[a] Half-Maximal Inhibition			
	−IFN-γ[b]		+IFN-γ	
	TNF	LT	TNF	LT
L929	2,30	600		
BT-20			~6,000	~30,000
HT-29	1,000	3,000	1,000	2,000
SK-CO-1	180	350	180	450

[a] Concentration of unlabeled TNF or LT required to displace one-half of the bound ^{125}I-TNF from the cells.
[b] The cells were incubated with or without IFN-γ for 6 h before performing the binding assay.

ity receptors and although accurate data could not be obtained, clearly much higher levels of TNF and LT were required to displace labeled TNF from receptor sites on this cell. Interferon treatment had no effect on the ability of cold ligand to displace labeled TNF, again agreeing with the lack of modulation of receptor affinity by IFN. In these experiments, the relative binding affinities of TNF for these lines as defined by the 50% inhibition point roughly paralleled the antiproliferative effects, however, the concentrations were generally at least 10-fold higher than the level yielding 50% of the biologic effect. In a classic analysis of various agonists competing for the same receptor, the binding curves can be shifted reflecting unequal receptor affinities, yet they should be parallel. In this logit analysis, the TNF curve for the SK-CO-1 line was found to be nonlinear and had a different slope when compared with the LT binding curve. The quality of the HT-29 data do not allow one to define accurately the curve shape. This observation indicates that the interactions of these two ligands with the receptor are not identical. Looking at the TNF-binding curve with the SK-CO-1 line (Fig. 6), one could surmise that the curves were roughly parallel at high ligand concentrations, yet deviate in the low concentration regions. The nonlinearity in the TNF curve may reflect the receptor heterogeneity seen in the Scatchard analysis. It should be noted that radiolabeled TNF binding studies may be plagued by an additional problem. Iodination by methods such as iodogen that yield high specific activity labeling do lead to TNF inactivation when the labeling is allowed to continue for longer times (J. Browning, unpublished observations). It is possible that individual subunits of the TNF trimer may be inactivated without serious loss of biologic activity, i.e., less than 50 to 70% losses. These partially defective yet more highly iodinated TNF molecules may exhibit abnormal binding in Scatchard analyses.

DISCUSSION

We have shown in this study that TNF and LT have different antiproliferative properties on several human tumor lines. The concentration of LT necessary for a half maximal antiproliferative effect remained in the range of 200 to 400 pM for three tumor lines, whereas the TNF concentrations required for similar activity varied over two orders of magnitude. With few exceptions, the presence of only one class of TNF-binding site has been demonstrated with dissociati n constants ranging from 1 to 2000 pM depending upon the cell type. Unlabeled TNF and LT have been shown to compete with labeled TNF for binding to its receptor (28, 30) and vice versa, i.e., cold TNF could compete with labeled LT binding to its receptor (29, 31, 32). Typically, LT and TNF were almost equally potent in this respect. The simpliest mod l accommodating these data is the existence of a single receptor that can bind both LT and TNF. If one assumes that the biologic response is directly linked to receptor occupancy and that only a single type of TNF/LT receptor is present on these cells, it is difficult to explain the differing biologic effects of TNF and LT. In this case, the differing sensitivities to TNF may simply reflect the varying levels of signal transduction required to produce an effect, e.g., only 0.1 to 1% occupancy of the TNF receptors on a SK-CO-1 cell may be sufficient to trigger cytostasis. However, if LT is behaving as a simple TNF agonist, it too should trigger the biologic event at proportionately lower concentrations with a cell line like SK-CO-1. Because the potency of LT remained relatively unalter d in these different cells, more complex phenomena need to be considered.

Two possible explanations for this discrepancy have occurred to us. In the first case, both low and high affinity receptors for TNF exist with LT binding to a low affinity receptor with a K_d of about 10^{-10} to 10^{-9} M, but not to the high affinity form (K_d 10^{-10} to 10^{-12} M). In this model, signal transduction results from occupancy of either receptor form, however, the increased sensitivity of certain cell types is a consequence of TNF binding to the high affinity-receptor form. The high and low affinity forms may reflect either different states of the same receptor or different receptor molecules. In the second model, TNF binding to its receptor occurs with some form of positive cooperativity possibly stemming from its trimeric structure. The apparent high affinity binding would represent actually a high avidity binding mode of a common low affinity receptor. In this model, LT would be incapable of interacting in a cooperative fashion. Whether cooperative interactions occur would depend probably not only on the receptor density, but also on other factors such as cytoskeletal linkages, etc.

TNF-binding studies were undertaken in an attempt to resolve this dilemma and these results suggest that elements of both models may contribute. SK-CO-1 cells and probably HT-29 cells were found to possess a high affinity form of the TNF receptor correlating with the increases sensitivities of these lines to TNF. High levels of LT, that alone were inactive, were unable to affect th low level anti-proliferative effects of TNF with the SK-CO-1 line suggesting that LT cannot bind to the high affinity form. Furthermore, one could speculate that the high affinity form may be the biologically relevant TNF receptor and hence LT may not elicit TNF-like activities under physiologic conditions.

On the other hand, TNF was only three- to fivefold better than LT in competing with ^{125}I-TNF binding to the SK-CO-1 receptor in contrast to the 150- to 100-fold difference in biologic potencies. Thus the existence f a high affinity TNF receptor may explain the increased sensitivity of a cell type such as SK-CO-1 to TNF, however, the LT binding properties do not fit with its decreased biologic potency. It is possible that the competitive binding studies are complicated by the existence of a heterogenous receptor population. Although most studi s have n t detected high affinity TNF binding, there are

a number of exceptions. High affinity TNF binding (K_d 1 to 5 pM) was observed with human monocytes (51) and HL-60 cells (16, 52) and in the latter case the presence of the high affinity form correlated with increased TNF potency. Likewise, high affinity receptors with K_d in the 1 to 5 pM range for murine TNF were described on the mouse L cell (53). Intermediate affinity receptors in the range of 20 to 70 pM have been described on endothelial cells, lymphocytes, and the breast carcinoma line MCF-7 (23, 54–56). Evidence for multiple TNF receptors also comes from consideration of the species specificity of receptors on several murine cell lines. The murine L929 cell responds almost equally well to both human and murine TNF, yet the murine CT6 T cell line (57), the mouse/rat T cell hybridoma PC-60 (58), and the murine line PG-19 (59) respond to murine TNF but not to human TNF. Whether these phenomena reflect separate LT and TNF receptors is not clear.

The analysis of ligand binding to multiple receptors can be very complex, nonetheless, the data obtained in this study suggest an additional possibility. The logit plots of TNF and LT competition for labeled TNF binding should yield parallel lines if the two ligands interact in a similar manner with the same receptor albeit with different affinities. The lack of parallel plots may indicate some form of receptor cooperativity. Trimeric TNF could undergo multivalent interactions with TNF receptors leading to a high avidity TNF interaction. This interaction could allow for biologic effects at low concentrations despite low to moderate affinity TNF binding to its receptor. In this model, LT must be considerably less effective in forming multimeric interactions with this receptor form. In support, it was found that monomeric TNF was biologically less active and bound with a much lower affinity than trimeric TNF (41). Conversely, one study employing iodinated monomeric *E. coli* derived-human LT observed relatively high affinity binding to the L929 cell receptor (29). Although most TNF cross-linking studies have indicated the existence of a 55- to 75-kDa receptor chain, the molecular nature of the TNF receptor remains presently unresolved. Immunoaffinity-purified TNF receptor was found to undergo ligand-dependent oligomerization, possibly reflecting binding of one TNF trimer with several receptor molecules (60). An additional 138-kDa protein was associated with TNF-responsiveness in the MCF-7 carcinoma (54) and the possibility that this species resulted from TNF cross-linked dimeric forms should be considered. Resolution of these aspects awaits the cloning and characterization of the TNF/LT receptors.

LT and TNF pose a problem arising in other apparently redundant systems such as IL-1 types α and β and IFN-α and -β; namely, why do multiple forms of these cytokines exist? Although a region in the C-terminal domain that appears to be critical for receptor binding is relatively conserved (61), the amino acid sequences of LT and TNF are only 28% homologous overall (1, 2). The genes lie within 2 kb of each other (1), apparently the result of a gene duplication event, yet they are independently regulated (12, 13). Other than the disparate activities in the endothelial cell and monocyte systems and the antiproliferative effects described here, the qualitative biologic spectra of LT and TNF are quite similar. It is possible that the actual role of LT lies in an as yet undescribed system and relies on interaction with a LT-specific high affinity receptor. It was in this light that several groups have reintroduced the suggestion that LT may be a mediator of delayed type hypersensitivity reactions (26, 62). Whether nature enjoys the evolutional security of these redundant cytokines or exploits individual activities via separate receptors remains an open question.

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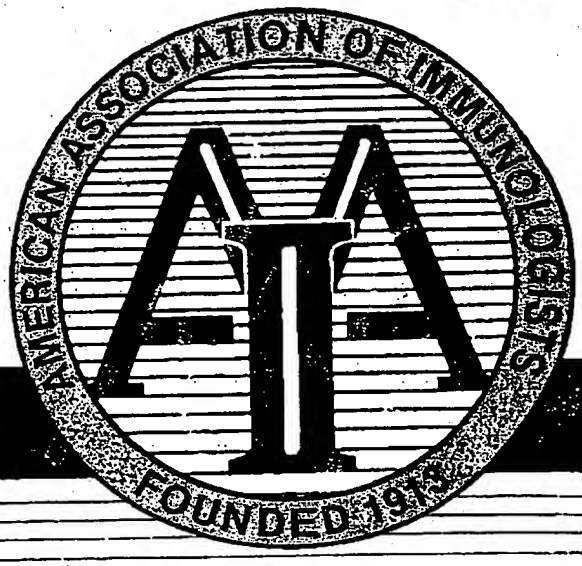

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